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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/06, C12P 21/08, C12N 5/20, C07K 15/00, G01N 33/577, 33/68

A1

(11) International Publication Number: WO 94/13795

(43) International Publication Date: 23 June 1994 (23.06.94)

(21) International Application Number: PCT/EP93/03499

(22) International Filing Date: 10 December 1993 (10.12.93)

(30) Priority Data:
92403403.6 14 December 1992 (14.12.92) EP
(34) Countries for which the regional or international application was filed: AT et al.

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(81) Designated States: AU, CA, JP, NO, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MONOCLONAL ANTIBODIES DIRECTED AGAINST THE MICROTUBULE-ASSOCIATED PROTEIN TAU, HYBRIDOMAS SECRETING THESE ANTIBODIES, ANTIGEN RECOGNITION BY THESE MONOCLONAL ANTIBODIES AND THEIR APPLICATIONS

(57) Abstract

The invention relates to a monoclonal antibody which forms an immunological complex with an epitope of an antigen belonging to normal human tau protein as well as abnormally phosphorylated human tau protein, with said tau protein being liable to be obtained from a brain homogenate, itself isolated from human cerebral cortex. The monoclonal antibodies of the invention can be used to detect tau and abnormally phosphorylated tau in brain extracts and in unconcentrated cerebrospinal fluid.

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MONOCLONAL ANTIBODIES DIRECTED AGAINST THE MICROTUBULE-ASSOCIATED PROTEIN TAU, HYBRIDOMAS SECRETING THESE ANTIBODIES, ANTIGEN RECOGNITION BY THESE MONOCLONAL ANTIBODIES AND THEIR APPLICATIONS

The invention relates to new monoclonal antibodies directed against the human microtubule-associated protein tau, to the hybridomas secreting these monoclonal antibodies, and to the antigen recognition by these monoclonal antibodies and their applications. The invention also relates to a process for diagnosing brain diseases involving the particular epitope (of the tau protein) which is recognized by said monoclonal antibodies.

Alzheimer's disease (AD) is the most common form of adult-onset dementia. At present, no biochemical test is available for antemortem diagnosis of AD. The disease is therefore clinically diagnosed primarily by exclusion of other forms of dementia. The illness is characterized neuropathologically by the presence of neuritic (senile) plaques and neurofibrillary tangles (NFT).

Neurofibrillary tangles consist of paired helical filaments (PHF), of which the main protein component is a modified form of the microtubule-associated protein tau (Brion et al., 1985; Greenberg and Davies, 1990; Lee et al.,1991), which under normal circumstances promotes microtubule assembly and stability (Weingarten et al., 1975; Bré and Karsenti, 1990), which is synthesized in the neurons of several species, including humans (Kosik et al., 1989) and which is abundantly present in the axonal compartment of these neurons (Binder et al., 1985).

The protein exists as a family of different isoforms of which 4 to 6 isoforms are found in normal adult brain but only 1 isoform is detected in fetal brain (Goedert et al., 1989). The diversity of the isoforms is generated from a single gene by alternative mRNA splicing (Himmler, 1989). The most striking feature of tau protein, as predicted from molecular cloning, is a stretch of 31 or 32 amino acids occurring in the carboxy-terminal part of the molecule that is repeated 3 or 4 times. Additional diversity is generated through 29 or 58 amino acid-long insertions in the NH_2-terminal part of the molecules (Goedert et al., 1989).

Tau variants of 64 and 69 kDa, which are abnormally phosphorylated, as revealed by the apparent increase in their molecular mass observed after alkaline phosphatase treatment, have been detected exclusively in brain areas showing neurofibrillary tangles and senile

plaques (Flament et al., 1989, 1990). The sites of phosphorylation by 4 different kinases have been mapped in the C-terminal microtubule-binding half of tau, and it could be shown that the action of a calcium calmodulin-dependent kinase on bacterially expressed tau resulted in the phosphorylation of Ser(405) which induced a lower electrophoretical mobility (Steiner et al., 1990). Tau present in paired helical filaments, called PHT-tau is abnormally phosphorylated (Lee et al., 1991). This abnormal phosphorylation causes a conformational change in tau, resulting probably in self-association and the formation of PHFs. PHF-tau in AD is phosphorylated at several sites, one of which is the phosphoserine 199 and/or 202. This site is specifically recognized by a mAb called AT8 (Biernat et al., 1992). Therefore, AT8 is a discriminative marker for PHF-tau (Goedert et al., 1992).

Several antibodies have been reported that show reactivity to human tau either because they are directed to non-specific phosphorylated epitopes present on neurofilament and subsequently shown to cross-react with normal and abnormally phosphorylated tau (Nukina et al., 1987; Ksiezak-Reding et al., 1987) or because they recognized specific epitopes on normal and abnormally phosphorylated tau (Kosik et al., 1988). In addition to the tau antibodies directed towards non-specific epitopes, antibodies directed specifically to phosphorylated tau epitopes have been described (Mercken et al., 1992b).

Although overall tau mRNA levels are only slightly modulated in Alzheimer-affected brain regions (Goedert et al., 1988; Barton et al., 1990) it has been shown that total tau protein levels may differ at least 6-fold (Khatoon et al., 1992). This has been demonstrated by polyclonal antibodies against tau (Flament and Delacourte, 1990) and by monoclonal antibodies directed to well-defined epitopes. The Alz 50 monoclonal antibody recognizing a phosphate-independent epitope in the N-terminus of the tau molecule (Goedert et al., 1991) has been used in a sandwich immunoassay on brain homogenates and it has been shown that tau levels are higher in Alzheimer's patient brains (Ghanbari et al., 1990; patent application EP 444 856).

An antibody named "423", raised against pronase-treated PHFs and specifically reactive with a 9.5 kDa and a 12 kDa fragment was also used to measure tau protein in Alzheimer's disease (patent application WO 89/03993). Similarly, it was found that increased mAb 423 immunoreactivity was observed in Alzheimer brain homogenates as compared with control brain homogenates.

Mercken et al. (1992b) describe a range of monoclonal antibodies which are either

specific for a phosphatase-sensitive epitope (AT8) or which react with PHF-tau as well as with normal tau (AT1, AT4, AT6, AT9, AT11, AT12 and AT14) in Western blotting.

Moreover, the antibody tau 1 (Wischik et al., 1988; Harrington et al., 1990) was also used to measure tau in brain homogenates. In one case when tau levels were specifically measured in Alzheimer-affected brain sections, tau levels were eight-fold higher as compared with levels in normal brain homogenates (Khatoon et al., 1992).

In a first attempt to diagnose Alzheimer disease in cerebrospinal fluid, the PHF-tau-specific monoclonal antibody AT8 (Mercken et al., 1992b), was used. However, no PHF tau antigen could be demonstrated.

Thus far, none of the monoclonals that have been described have been successful in detecting tau in non-concentrated cerebrospinal fluid (CSF), although the presence of tau was observed in 100-fold concentrated CSF (Wolozin and Davies, 1987) or in CSF samples using polyclonal antibodies (Delacourte and Vermersch, 1991).

The aim of the present invention is therefore to provide monoclonal antibodies which allow reliable and sensitive detection of normal and abnormally phosphorylated tau present in brain extracts and in unconcentrated cerebrospinal fluid. The invention also aims at providing the hybridoma which secretes the above-said monoclonal antibodies.

The invention furthermore aims at providing the epitope of the tau protein present in brain homogenates or in body fluids such as cerebrospinal fluid, which is recognized by said monoclonal antibodies.

The invention aims at providing a process for the detection or diagnosis *in vitro* of brain diseases involving tau protein.

The monoclonal antibodies of the invention are characterized by the fact that they react with an epitope which is present in both normal and abnormally phosphorylated human tau protein. The monoclonal antibodies are furthermore characterized by the fact that they form an immunological complex with an epitope or an antigen belonging to normal and abnormally phosphorylated human tau protein. The monoclonal antibodies of the invention are also characterized by the fact that they do not form an immunological complex with other phosphorylated proteins such as MAP-1, MAP-2 and neurofilaments which share part of their sequence with tau protein (Nukina et al., 1987; Lewis et al., 1988) as determined by means of an ELISA. The monoclonal antibodies of the invention are also characterized by the fact that they are liable to detect human tau protein in CSF, with said tau protein being at a

concentration as low as 1.0 pg/ml and with said tau protein being detected at 100% recovery upon the addition of a fixed amount of tau protein in tau protein-negative CSF (100% spiking recovery).

The monoclonal antibodies of the invention also enable the diagnosis of Alzheimer's disease (AD) on the basis of CSF, i.e., to detect tau and modified forms of tau in CSF. The problem associated herewith is that this antigen is present in a very low amount in CSF, therefore the detection assay must be very sensitive. This problem can be resolved by using the combination of the monoclonal antibody of the invention together with the catalysed reporter deposition amplification technique (CARD, Bobrow et al., 1989), allowing a tau-specific CARD ELISA with a higher sensitivity. Alternatively, a mixture or combinations of labeled monoclonal antibodies, each recognizing epitopes different from AT120 epitope, could be used as detector antibodies.

The results obtained with the monoclonal antibody secreted by the hybridoma AT120 of the invention indicate that elevated tau levels are not only found in AD, but also in other neurological diseases where neuronal death or damage occurs.

The expression "form an immunologically complex with" means that the monoclonal antibody of the invention binds to the above-said antigen under one of the following conditions as mentioned in the techniques below:

- Light immunomicroscopy

Brain tissue samples, obtained at surgery or autopsy, are fixed by immersion in 4% formalin or Bouin's fixative and embedded in paraffin for sectioning. The monoclonal antibodies of the invention are applied in conjunction with a technique to visualize the formed immune complexes such as the avidin-biotinylated peroxidase complex technique (Hsu et al., 1981) using 3,3'-diaminobenzidine tetrahydrochloride for development of color. Sections are counterstained with Harris haematoxylin stain.

- Immunoelectron microscopy in tissue sections

Brain tissue samples, obtained at surgery or autopsy are fixed in either Bouin's fixative or 10% buffered formalin before sectioning without embedding (Vibratome). The monoclonal antibody of the invention is used for immunostaining by the indirect immunogold method after which the sections are fixed, embedded and sectioned for electron microscopy, all according

to standard protocols known to those skilled in the art (Brion et al., 1985).

- Immunoblotting procedures

For immunoblotting, fractions enriched in tau are prepared as described (Lindwall and Cole, 1984). Typically, 50 g of brain tissue is cut into small pieces with scissors and homogenized 1:1 (wt/vol) in buffer A (20 mM 2-[N-morpholino]ethanesulfonic acid, 80 mM NaCl, 2 mM EDTA, 0.1 mM EGTA, 1 mM ß-mercaptoethanol, pH 6.75) with a Potter homogenizer equipped with a Teflon plunger. The homogenate is centrifuged for 1 h at 150,000 g at 4°C, and the supernatant is heated for 5 min in boiling water and chilled again for 10 min on ice. The slurry is centrifuged for 2 h at 150,000 g at 4°C, and the supernatant is collected. The heat-stable cytosolic extract is added to 2.5% perchloric acid and centrifuged for 1 h at 150,000 g at 4°C, after which the supernatant is neutralized with 3 M Tris. The supernatant is then dialyzed in water and concentrated in a Centriprep concentrator (Amicon, Lausanne, Switzerland).

SDS-polyacrylamide electrophoresis is performed under reducing conditions on 12% gels (Laemmli, 1970). After electrophoresis, the proteins are either fixed and stained with Coomassie brilliant blue, or transferred (Towbin et al., 1979) to nitrocellulose sheets (Hybond-C, Amersham) or Immobilon filters (Millipore).

After transfer, the filters are presoaked in PBS containing 0.05% (v/v) Tween 20 (Tween-PBS) and then incubated for 1 h in Tween-PBS containing 5% (w/v) skimmed dried milk and 10% (v/v) newborn calf serum (blocking buffer). Next, the filters are treated overnight at 4°C with a monoclonal antibody according to the invention appropriately diluted in blocking buffer.

The filters are then washed three times in Tween-PBS and treated for 1.5 h at room temperature with horseradish peroxidase-labeled rabbit anti-mouse IgG (Dakopatts, Denmark) diluted 1/3000 in blocking buffer. After three washes in Tween-PBS, streptavidine-biotinylated horseradish peroxidase complex (Amersham), diluted 1/250 in blocking buffer, is applied for 1.5 h at room temperature. Thereafter, the filters are washed three times in Tween-PBS and once in PBS. The filters are then incubated in PBS containing 0.05% (w/v) diaminobenzidine and 0.03% (v/v) hydrogen peroxide until background staining develops.

It should be clear that the formation of an immunological complex between the

monoclonal antibodies and the antigen is not limited to the precise conditions described above, but that all techniques that respect the immunochemical properties of the antibody and antigen binding will produce similar formation of an immunological complex.

Human normal tau is a class of at least six tau proteins ranging in molecular weight from 58 to 64 kDa which are specifically expressed in the somatodendritic domain of all neurons (Papasozomenos and Binder, 1987). Moreover Alzheimer (tangle)-specific tau forms have been described which occur in the degenerating cortical neurons of Alzheimer's disease or Down's Syndrome and of which the lower electrophoretic mobility can be attributed to abnormal phosphorylation (Flament et al., 1989; Delacourte et al., 1990).

According to an advantageous embodiment of the invention, the monoclonal antibody forms an immunological complex with all forms of tau described above, with said human tau protein being liable to be obtained from a brain homogenate, itself isolated from the cerebral cortex of a patient suffering from a neurological disease.

A "brain homogenate" and tau protein can be obtained by the man skilled in the art according to standard methods such as the method of Lindwall and Cole (1984).

According to an advantageous embodiment, the monoclonal antibodies of the invention form an immunological complex:

- either with an epitope located within the following amino acid sequence of human tau protein :

NH_2- Arg Gly Ala Ala Pro Pro Gly Gln Lys Gly Gln

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    155                 160                 165
Ala Asn Ala Thr Arg Ile Pro Ala Lys Thr Pro Pro
                170                 175
Ala Pro Lys Thr Pro Pro Ser Ser Gly Glu Pro Pro
        180                 185
Lys Ser Gly Asp Arg Ser Gly Tyr Ser Ser Pro Gly
190                 195                 200

Ser Pro Gly Thr Pro Gly Ser Arg Ser Arg Thr Pro
            205                 210
Ser Leu Pro Thr  Pro Pro Thr Arg                (SEQ ID NO 1)
    215                 220
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- or with any other peptide capable of forming an immunological complex with a monoclonal antibody, which itself is liable to form a complex with the epitope located in the tau protein region as shown in SEQ ID NO 1.

The sequence as shown in SEQ ID NO 1 will be hereafter designated as containing "the epitope" of the invention. Said amino acids sequence spans the amino acid 155-221 of human tau using the numbering of human tau 40 (Goedert et al., 1989).

The peptides capable of forming an immunological complex with a monoclonal antibody, which itself is liable to form a complex with the above-mentioned peptide will be defined as the "variant peptides".

A preferred monoclonal antibody of the invention is secreted by the hybridoma deposited at ECACC (European Collection of Animal Cell Cultures, Vaccine Research and Production Laboratory, Public Health and Laboratory Service (PHLS), Centre for Applied Microbiology and Research, Proton Down, GB-Salisbury, Wiltshire SP4 OJG) on October 8, 1992 under No. 92100853.

This hybridoma will be hereafter designated as "hybridoma AT120" and the secreted monoclonal antibody will be designated as "monoclonal antibody AT120".

The invention also relates to the hybridoma which secretes a monoclonal antibody according to the invention, and particularly the hybridoma filed at ECACC on October 8, 1992 under No. 92100853.

The above-mentioned monoclonal antibodies are obtained by a process involving obtention and isolation of hybridomas which secrete these monoclonal antibodies.

A process for obtaining the hybridoma involves:

- starting from spleen cells of an animal, e.g. mouse or rat, previously immunized in vivo or from spleen cells of such animals previously immunized *in vitro* with an antigen recognized by the monoclonal antibodies of the invention, such as the monoclonal antibody secreted by the hybridoma deposited at ECACC on October 8, 1992 under No. 92100853;

- fusing said immunized cells with myeloma cells under hybridoma-forming conditions; and

- selecting those hybridomas which secrete the monoclonal antibodies which specifically recognize an epitope of the above-said antigen and which form an immunological complex with normal tau or the abnormally phosphorylated form of tau protein or with the

peptide comprising the epitope of tau recognized by the monoclonal antibody of the invention.

A process for producing the corresponding monoclonal antibodies involves:

- culturing the selected hybridoma as indicated above in an appropriate culture medium: and

- recovering the monoclonal antibodies secreted by said selected hybridoma: or alternatively

- implanting the selected hybridoma into the peritoneum of a mouse and, when ascites has been produced in the animal:

- recovering the monoclonal antibodies then formed from said ascites.

The monoclonal antibodies of the invention can be prepared by conventional *in vitro* techniques such as the culturing of immobilized cells using e.g. hollow fibers or microcapsules or such as the culturing of cells in homogeneous suspension using e.g. airlift reactors or stirred bioreactors.

The invention also relates to a peptide (antigen), which can be obtained from a human brain homogenate itself being isolated from the human cerebral cortex obtained from a patient having Alzheimer's disease, and which forms an immunological complex with the monoclonal antibody of the invention.

The invention also relates to peptides (antigens) which are liable to form an immunological complex with anyone of the monoclonal antibodies of the invention and

- which are contained in or are constituted by parts of the sequence as shown in SEQ ID NO 1;

- which contain or are constituted by the sequence of the variant peptides defined above.

It is to be recalled that variant peptides are those peptides able to form an immunological complex with a monoclonal antibody, which itself is liable to form a complex with an epitope located in the tau protein region as shown in SEQ ID NO1.

The invention also relates to polypeptides (antigens) of about 100 amino acids

- which contain the sequence as shown in SEQ ID NO 1, or

- which contain the sequence of the variant peptides defined above.

The invention also relates to the above- mentioned peptides which are liable to generate monoclonal antibodies of the invention.

The invention also relates to a peptide (antigen) which is contained in the brain, in

the cerebrospinal fluid, or in the serum of a patient having Alzheimer's disease or any brain disease involving normal human tau protein and which forms an immunological complex with a monoclonal antibody of the invention.

The invention also relates to a peptide (antigen) which is contained in the brain, in the cerebrospinal fluid, or in the serum of a patient having Alzheimer's disease or any brain disease involving PHF or abnormally phosphorylated human tau protein and which forms an immunological complex with a monoclonal antibody of the invention.

A method for preparing the peptides of the invention is characterized in that, preferably starting from the C-terminal amino acid, the successive aminoacyls in the requisite order, or aminoacyls and fragments formed beforehand and already containing several aminoacyl residues in the appropriate order, or alternatively several fragments prepared in this manner beforehand, are coupled successively in pairs, it being understood that care will be taken to protect all the reactive groups carried by these aminoacyls or fragments beforehand except for the amine groups of one and carboxyl groups of the other, or vice versa, which must normally participate in peptide bond formation, in particular after activation of the carboxyl group, according to methods known in peptide synthesis, and so on, proceeding stepwise up to the N-terminal amino acid.

The antigen of the invention, which can be prepared by methods known to those skilled in the art (Lindwall and Cole, 1984) starting from the human cerebral cortex is characterized by its ability to form an immunological complex with the monoclonal antibody of the invention as defined above, advantageously with the monoclonal antibody secreted by the hybridoma AT120 deposited at the ECACC under No. 92100853 on October 8, 1992.

The antigen of the invention is advantageously contained in the brain, in the cerebrospinal fluid or the serum of a patient having Alzheimer's disease, Down syndrome, Pick's disease, subacute sclerosing panencephalitis (SSPE) or other neurological diseases in which the normal tau or abnormally phosphorylated tau protein are implicated: this antigen provokes an immunological reaction with the monoclonal antibody of the invention.

The invention also relates to a process for the detection or the diagnosis *in vitro* of brain disease involving tau protein, i.e. Alzheimer's disease, which involves:

- bringing the monoclonal antibody of the invention into contact with a preparation of NFT containing tau protein or a detergent-extracted brain homogenate containing tau protein isolated from a patient having had Alzheimer's disease or any other disease involving tau

protein or abnormally phosphorylated tau protein under conditions suitable for producing an antigen-antibody complex; and,

- separating the antigen from said complex and recovering the antigen sought in a purified form.

The preparation of tau can be carried out according to Lindwall and Cole (1984).

Advantageously, the monoclonal antibodies used are in an immobilized state on a suitable support such as a resin. The process for the detection of the antigen can then be carried out as follows:

- bringing the supernatant containing proteins and polypeptides obtained as a result of an extraction procedure starting from brain tissues or cerebrospinal fluid known to those skilled in the art (Iqbal et al., 1984; Greenberg and Davies, 1990) into contact with said monoclonal antibody, under such conditions as to allow the formation of an immunological complex;

- washing the immobilized antibody-antigen complex then formed;

- treating this complex with a solution (e.g. 3 M potassium thiocyanate, 2.5 M magnesium chloride, 0.2 M citrate-citric acid, pH 3.5 or 0.1 M acetic acid) capable of producing the dissociation of the antigen- antibody complex; and;

- recovering the antigen in a purified form.

The process of the invention for the detection or diagnosis *in vitro* of brain disease involving tau protein and abnormally phosphorylated tau protein, as e.g. in Alzheimer's disease, includes:

- bringing a sample of a brain homogenate, or of cerebrospinal fluid, or of serum from a patient suspected of suffering from a neurological disorder involving tau protein or PHF, more particularly Alzheimer's disease, into contact under *in vitro* conditions with the monoclonal antibody of the invention, with said conditions being suitable for producing an antigen-antibody complex; and

- detecting the immunological binding of said antibody to said sample of brain homogenate, or of cerebrospinal fluid, or of serum.

The detection of the immunologically bound monoclonal antibody can be achieved by conventional technology. Advantageously, the monoclonal antibody of the invention itself carries a marker or a group for direct or indirect coupling with a marker as exemplified hereafter. Also, a polyclonal antiserum can be used which was raised by injecting the antigen

of the invention in an animal, preferably a rabbit, and recovering the antiserum by immunoaffinity purification in which said polyclonal antibody is passed over a column to which said antigen is bound and eluting said polyclonal antibodies by conventional technology.

Detection can also be achieved by competition binding of the antigen with a labeled peptide comprising the epitope of the invention.

A particularly advantageous embodiment of the process of the invention for the detection or diagnosis *in vitro* of brain diseases involving PHF and/or normal tau protein, e.g. Alzheimer's disease, comprises the steps of:

- bringing a sample of unconcentrated cerebrospinal fluid sample isolated from a patient suspected of suffering from a neurological disorder involving normal or abnormally phosphorylated tau protein, more particulary Alzheimer's disease, into contact under *in vitro* conditions with a monoclonal antibody according to the invention, under conditions suitable for producing an antigen-antibody complex;

and,

- detecting the immunological binding of said antibody to said sample of cerebrospinal fluid by means of a sandwich ELISA, preferably by applying the catalysed reporter diagnosis enhancement (CARD) procedure.

The invention also relates to a kit for the diagnosis *in vitro* of one of the following diseases: Alzheimer's disease, Down's syndrome, Pick's disease, subacute sclerosing panencephalitis (SSPE) and other neurodegenerative disorders in which normal tau protein or abnormally phosphorylated tau protein are implicated. Such a kit would contain:

- at least one microplate for deposition thereon of any monoclonal antibody of the invention;

- a preparation containing the sample to be diagnosed *in vitro*, possibly together with a labeled peptide containing the epitope of the invention and preferably, a peptide lying in the peptide sequence as shown in SEQ ID NO 1.

- a second antibody

* which can be a monoclonal antibody recognizing another epitope of normal or abnormally phosphorylated tau protein, or of any peptide of the invention, with said epitope being different from the one of the invention, or

* which can be a polyclonal antibody directed against normal or abnormally

phosphorylated tau, or against a peptide of the invention, with said polyclonal antibody being liable to form an immunological complex with epitopes which are all different from the epitope of the invention, with said polyclonal antibody being preferably purified by immunoaffinity chromatography using immobilized tau protein, or

- a marker either for specific tagging or coupling with said second antibody;

- appropriate buffer solutions for carrying out the immunological reaction between the monoclonal antibody of the invention and a test sample on the one hand, and the bound second antibody and the marker on the other hand.

The labeled peptide mentioned above can be a peptide which has been labeled by any means known for the man skilled in the art. Moreover, the marker specific for tagging and coupling can be any marker known to the man skilled in the art.

The invention also relates to a kit, as described above, also containing the antigen of the invention, with said antigen of the invention being either a standard (for quantitative determination of the antigen which is sought) or a competitor, with respect to the antigen which is sought, for the kit to be used in a competition dosage process.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1

Western blotting detection of normal tau or PHF-tau using the monoclonal antibodies Tau-1, (Binder et al., 1985) AT8 (Mercken et al., 1992b) and AT120. Lanes 1, 3 and 5: PHF-tau isolated according to Greenberg and Davies (1990). Lanes 2, 4 and 6: Normal affinity purified human tau according to Mercken et al. (1992a). Lane 7: Molecular weight markers.

Lanes 1 and 2 were developed using AT8, lanes 3 and 4, using AT120 and lanes 5 and 6, using Tau-1 monoclonal antibody.

Figure 2A and Figure 2B

Detection of tau protein by immunochemistry

Fig. 2A: section from hippocampus of a patient with Alzheimer's disease. Magnification 212 x.

Fig. 2B: section from another hippocampus of a patient with Alzheimer's disease with

abundant tangles. Magnification 212 x.

Figure 3

Titration of normal (0) and PHF tau (■), spiked in a tau-negative CSF pool using the amplified (CARD) AT120 sandwich ELISA. All dilutions were tested in duplicate and the data presented as optical density (OD) units.

Figure 4

Western blotting of several deletion mutants constructed as indicated in example V, and stained with AT120 as indicated in Example I. Mutants comprise the following amino acids (AA); Lane 1, full length tau 34 (Goedert et al., 1989); Lane 2, amino terminus of tau 34 up to AA_{154}; Lane 3, from AA_{155} to the carboxyterminus of tau 34; Lane 4; aminoterminus of tau 34 up to AA_{242}; Lane 5, aminoterminus tau 34 up to AA_{221}; Lane 6, from AA_{222} to the carboxyterminus of tau 34.

Figure 5

Epitope recognition sites of the monoclonal antibodies HT7, BT2, AT8 are depicted on the epitope of the invention (SEQ ID NO 1) shown in the one letter amino acid code. Epitopes are boxed. The star "*" designates the fact that the AT8 epitope recognition needs phosphorylation of serine residue 202.

EXAMPLES

Example I: Preparation of the monoclonal antibody AT120 (IgG1, subtype kappa)

1. Preparation of the antigen for immunization

PHF-tau was partially purified by a modification of the method of Greenberg and Davies (1990). Postmortem tissue, consisting mostly of gray matter from the frontal and temporal cortex, was obtained from histologically confirmed Alzheimer patients. This Alzheimer gray matter brain sample (5-10 g) was homogenized with 10 volumes of cold buffer H (10 mM Tris/1 mM EGTA/0.8 M NaCl/10% sucrose, pH 7.4) in a Teflon/glass

Potter S (Braun, Germany) homogenizer. After centrifugation of the homogenate in a 60 Ti MSE rotor at 27,000 x g for 20 min at 4°C, the pellet was removed and the supernatant was adjusted to 1% (wt/vol) N-laurosylsarcosine and 1% (vol/vol) 2-mercaptoethanol and incubated while rotating on a mixer (Swelab, Sweden) for 2.5 hours at 37°C. The supernatant mixture was centrifuged at 108,000 x g for 35 min at 20°C. The PHF-tau containing pellet was gently washed with PBS and finally suspended in 1 ml of the same buffer.

The antigen preparation was evaluated by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blotting using immunoblotting with polyclonal rabbit antihuman normal tau antiserum (Mercken et al., 1992b).

2. Immunization protocol and fusion procedure

Balb/c mice were primed subcutaneously with 100 μg partially purified PHF-tau in complete Freund's adjuvant and boosted intraperitoneally 3 times thereafter at 3-week intervals with 100 μg of the same antigen in incomplete Freund's adjuvant. On days 3 and 2 before the fusion, mice were boosted with 100 μg PHF-tau in saline.

Mouse spleen cells were fused with SP2/0 myeloma cells, using a modified procedure of Köhler and Milstein (1975), with PEG 4000.

The cells of the fusion experiment were suspended at a density of 4.5 x 10^4 spleen cells/well on 96-well plates preseeded with mouse peritoneal macrophage cells as a feeder layer. These wells were screened after 12 days for anti-tau antibody production in a sandwich ELISA either specific for normal tau or for PHF-tau as discussed in section 3. below.

Hybridoma growth was in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum, sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), and nonessential amino acids. All products were purchased from Gibco, (Paisley, U.K.). Cells were incubated in a humidified CO_2-air incubator.

3. Sandwich ELISA for antibody screening

The screening ELISA used for the detection of anti-tau monoclonal antibodies was

a sandwich ELISA system with affinity-purified polyclonal rabbit anti-human tau antibodies (Mercken et al; 1992a) in the coating phase. To this end, purified human normal tau, prepared as described in Mercken et al. (1992a) was used for the preparation of an immuno-affinity column using cyanogen bromide-activated Sepharose (Pharmacia, LKB Sweden). The affinity-bound anti-tau fraction was eluted from this column with a 0.1 M citric acid buffered solution at pH 2.5. After neutralization, the anti-tau-containing fractions were pooled and coated overnight (1 μg/ml) at 4°C on high-binding microtiter plates (Nunc, Gibco, Paisley, UK) in coating buffer (10 mM Tris, 10 mM NaCl, 10 mM NaN_3, pH 8.5). After overcoating for 30 min with 125 μl 10%-saturated casein in PBS to reduce non-specific binding, the plates were incubated with 100 μl of an appropriately diluted PHF-tau preparation and incubated for 60 min at 37°C. The plates were washed 3 times with PBS-0.05% Tween 20 (v/v); 100 μl hybridoma supernatant was added and incubation was continued for 1 h at 37°C. After washing, the bound monoclonal antibodies were detected with peroxidase-conjugated rabbit anti-mouse serum (Dakopatts, Glostrup, Denmark). All reagents were diluted in PBS with 10% casein. After final washing, 100 μl 0.42 mM 3,5,3',5'-tetramethylbenzidine, 0.003% H_2O_2 v/v in 100 mM citric acid, 100 mM disodium hydrogen phosphate, pH 4.3, was added as peroxidase substrate. The reaction was stopped with 50 μl of a 2 M H_2SO_4 solution. Absorbance was read in a Titertek Multiscan (Flow Laboratories, Eflab, Oy, Finland) at 450 nm.

The cross-reactivity of the monoclonal antibodies with normal tau in ELISA was tested in a sandwich ELISA identical to the screening assay, except that affinity-purified normal tau was used as the antigen instead of PHF-tau.

At the first selection of positive hybridoma cultures, most positive cultures were initially composed of mixed clones as seen by visual inspection of the wells (3-4 clones per well). These positive cultures were arbitrarily designated AT1 to AT24 (some of these hybridoma cultures, i.e., AT1 to AT14 were described by Mercken et al., 1992b). After this initial screening round, hybridoma cultures were subcloned by limiting dilution, a technique well-know to those skilled in the art, finally resulting in pure hybridoma clones secreting antibodies with a homogeneous idiotype. These pure hybridoma clones were further tested with respect to their reactivity pattern on normal and PHF-tau in ELISA, Western blotting and immunohistochemistry, and to their capability to diagnose neurological diseases by means of their affinity for tau protein present in an undiluted sample of cerebrospinal fluid. Based

on these criteria, the monoclonal antibody AT120 was selected and further characterized as shown in the following examples.

4. Determination of the antibody class and subclass

The antibody class and subclass was determined by Inno-LIA (Innogenetics, Ghent, Belgium). The antibody of the invention, AT120, appeared to be of the IgG1, kappa subtype.

Example II: Detection of pathological tau and normal tau in ELISA and by Western blotting

1. Detection of normal tau in ELISA using AT120.

Protein G-purified monoclonal antibody AT120, obtained from serum-free hybridoma AT120 conditioned medium, was coated on ELISA plates and reacted with different dilutions of affinity-purified human normal tau as described in Mercken et al. (1992a), prepared in a solution of PBS and 10% casein.

The purity of normal tau was determined by SDS-PAGE. Tau samples were analyzed on an 420 A/H amino acid analyzer (Applied Biosystems B.V., Maarssen, The Netherlands) according to the manufacturer's instructions and the protein showed the expected amino acid composition. From the amino acid composition and by comparison with a standard peptide the concentration of normal tau was determined.

After incubation of the ELISA plates with different concentrations of tau spiked in tau- and PHF-tau-negative CSF for 1 h at room temperature, the plates were washed and incubated with 0,2 μg/ml biotynilated BT2 and HT7, each recognizing an epitope different from the AT120 epitope and present on normal term. After washing, complexed biotynilated antibodies were detected with horseradish peroxidase conjugated streptavidine (Jackson) and color development as specified in example I. The results are shown in Table I and Figure 3.

TABLE I. Detection of normal tau in ELISA

CONCENTRATION (pg/ml)	ABSORBANCE (expressed as milliabsorbance units) PHF-tau	normal tau
160	1682	1609
80	901	970
40	566	678
20	257	256
10	143	154
0	92	87

2. Detection of pathological tau and of normal tau in Western blotting using AT120.

Purified normal human tau and PHF-tau were applied to 10% SDS-polyacrylamide gels and run under denaturing conditions according to Laemmli (1970). After SDS-PAGE, the transfer to nitrocellulose (Hybond-C, Amersham, Brussels, Belgium) was carried out in 10 mM $NaHCO_3$, 3 mM Na_2CO_3, pH 9.9 for 120 min at 55 V with cooling. After blotting, the nitrocellulose was equilibrated to phosphate buffered saline (PBS), and protein binding sites were blocked with blot buffer (PBS supplemented with 5% w/v skimmed dried milk and 10% v/v newborn calf serum). Blotted proteins were incubated overnight at 4°C with AT120 as primary antibody. After 3 washings with PBS-0.05% Tween 20(v/v), horseradish peroxidase-labeled rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) were used at a dilution of 1/3000 and were incubated for 90 min at room temperature. All antisera were diluted in blot buffer. The blots were then washed three times in PBS/Tween and developed with substrate solution (PBS,0.05% w/v 3,3'-diaminobenzidine, 0.03% v/v H_2O_2) after which the reaction was stopped in H_2O. Results shown in Fig. 1 indicate that the AT120 antibody recognizes all tau isoforms. By contrast, the Tau-1 antibody (Binder et al., 1985)

reacts solely with normal tau, and the AT8 antibody (Mercken et al., 1992b) only with PHF-tau.

AT120, AT8 and Tau-1 mAbs were tested for phosphatase sensitivity of their epitopes in ELISA and in Western Blot on PHF-tau antigen. The reactivity of the AT120 antibody with PHF tau was not sensitive to phosphatase treatment either in ELISA (data not shown) or on Western blots (data not shown). The reactivity of AT8 was almost completely abolished after alkaline phosphatase treatment of the PHF-tau antigen in ELISA. Dephosphorylation of PHF-tau enhanced Tau-1 immunoreactivity, as described previously (Binder et al., 1985).

Example III: Detection of tau by immunohistochemistry

Paraffin sections of formalin-fixed brain tissue from neocortex, hippocampus, cerebellum, pons, and spinal cord of several Alzheimer patients and age-matched controls were prepared, as well as sections of peripheral nerve from one control patient.

Cryostat sections from Alzheimer and age-matched control brain were also prepared. Tissues were immunostained either with the peroxidase-antiperoxidase (PAP) technique (Steinberger et al., 1970) or with the avidin-biotin complex (ABC) technique (Hsu et al., 1981) using Dakopatts (Denmark) and Amersham (UK) reagents, respectively.

Briefly, after blocking non-specific interactions with normal swine serum (Dakopatts X901) diluted 1:25 in Tris-buffered saline (TBS) containing 1 % bovine serum albumin (BSA), sections were incubated overnight with the AT120 primary antibody appropriately diluted in TBS/BSA. Secondary antibody and peroxidase complex were then applied for 30 min each, with intermediate rinsing in TBS. Color was developed with 3,3'-diaminobenzidinetetrahydrochloride (Sigma). Sections were counterstained with Harris' hematoxylin, dehydrated, coverslipped, and viewed under a light microscope.

Figures 2A and 2B clearly indicate that AT120 produces abundant staining of NFT, dystrophic neurites in plaques, and dispersed staining of neuropil (neuropil threads).

Example IV: Detection of tau in cerebrospinal fluid samples

Cerebrospinal fluid samples

CSF samples from patients were collected at the Department of Neurology of the University Hospital of Antwerp. All samples were obtained by lumbar puncture performed

for routine diagnostic purposes. CSF samples were frozen and kept at -75°C in small aliquots until use.

The patients were divided into 3 different groups: 27 patients diagnosed with probable AD according to Mc Khann et al. (1984), mentally healthy control patients, who underwent lumbar puncture for radiculopathy and patients suffering from other neurological diseases (OND). The OND group included inflammatory, vascular, and other diseases, including patients with neurodegenerative diseases such as adenoleukodystrophy, frontal lobe degeneration, cerebellar atrophy, olivo-ponto-cerebellar atrophy, and amyotrophic lateral scelerosis. The age, sex and diagnosis were noted for each patient.

AT120 assay

AT120 monoclonal antibodies purified from serum-free conditioned medium by Protein G column chromatography were coated overnight at 4°C on high-binding microtiter plates (Nunc, Gibco, Paisley, UK) in coating buffer at 3 μg/ml (10 mM Tris, 10 mM NaCl, 10 mM NaN_3, pH 8.5). After overcoating for 30 min with 150 μl 10%-saturated casein in PBS to reduce non-specific binding, the plates were incubated with 25 μl CSF and 75 μl conjugate mixture containing 0.2 μg/ml of biotynilated BT_2 and an equal amount of HT7 in 5% Tween 20, 10% saturated casein in PBS. The plates were left overnight at room temperature and after washing peroxidase conjugated streptavidine (Jackson) (1/15000) was added for 30 minutes at room temperature.

Following an additional washing, 100 μl 0.42 mM 3.5, 3', 5' - tetramethylbenzidine 0.003% H_2O_2 v/v in 100 mM citric acid, 100 mM disodium hydrogen phosphate, pH 4.3, were added as perocidase substrate. The reaction was stopped with 50 μl of a $2MH_2SO_4$ solution. Absorbance was read in a Titertech Multiscan (Flow laboratories, Eflab, Oy, Finland) at 450 nm.

Absorbance values obtained with AT120 from the CSF samples were compared with standard curves generated from known quantities of affinity purified normal human tau and this comparison allowed the result to be expressed as pg tau/ml.

A summary of these results are compiled in Table II, where patients ID, diagnosis, age and tau values expressed in pg/ml CSF are listed. From these results, it is obvious that levels of control patients are substantially lower (mean : 16.4 pg/ml) as compared to the group of patients suffering from various neurological diseases (OND; mean value: 26.4

pg/ml). For patients with Alzheimer's disease the mean value is clearly elevated above those of control and OND samples (mean Alzheimer patient : 50.8 pg/ml).

If a cut-off level of 27 pg/ml is adapted, 8% of the control samples are positive, while for the OND group and the Alzheimer group these values are 27% and 80% respectively.

Table II : Mean Tau levels as determined with the AT120 ELISA assay, grouped according to control patients, Alzheimer patients (AD) and other neurological diseases (OND) and according to age cohhort.

Number	Diagnosis	Age	pg/ml	mean	Std
3	AD (early onset AD)	35	* 56.5	33.3	12.3
260	A.D.	41	* 31.2		
113	A.D.	44	* 42.6		
161	A.D. (possibly Crentzfeld)	57	* 33.8		
81	A.D.	58	* 14.5		
126	A.D.	59	* 24.8		
421	A.D.	59	* 29.8		
338	A.D.	64	* 51.2	61.68	35.4
254	A.D.	66	* 80.2		
209	A.D.	67	* 74.4		
383	Primary degenerative dementia	67	* 32.5		
38	A.D.	67	* 68.7		
229	A.D.	73	* 70.9		
132	A.D.	76	* 51.9		

88	A.D.	76	* 25.3		
65	Dementia	77	* 80.1		
71	A.D.	78	* 53.9		
28	A.D. (early onset A.D.)	78	* 48.7		
11	A.D.	85	* 14		
39	A.D.	86	* 150		
386	control	5	* 17.5	17.3	4.37
108	control	20	* 14.3		
402	control	26	* 14		
106	control	27	* 14		
424	control	28	* 14		
355	control	28	* 20.1		
399	control	29	* 14		
373	control	30	* 17.4		
381	control	31	* 14		
372	control	32	* 19.2		
379	control	32	* 18.3		
241	control	32	* 14		
415	control	32	* 14		
428	control	33	* 16.6		
118	control	34	* 21.5		
224	control	36	* 15.7		

24	control	37	* 25.2
369	control	38	* 23.9
425	control	39	* 14
145	control	40	* 31.9
61	control	40	* 17.3
377	control	40	* 14
366	control	41	* 17.7
401	control	41	* 20.1
400	control	42	* 14
417	control	42	* 14
354	control	43	* 16.8
34	control	43	* 25.8
217	control	43	* 14
364	control	44	* 14
134	control	45	* 16.7
367	control	45	* 14
427	control	45	* 14
394	control	48	* 14
361	control	48	* 14
237	control	50	* 17.2
396	control	52	* 16.4
100	control	54	* 23.9

411	control	54	* 14		
371	control	55	* 18.4		
423	control	56	* 14.9		
99	control	56	* 27.9		
192	control	60	* 19.9	24.83	15.17
387	control	61	* 14		
389	control	65	* 19.5		
426	control	66	* 15.4		
141	control	67	* 14		
368	control	67	* 15.4		
60	control	77	* 43.5		
348	control	80	* 56.9		
419	Hdrocephalus	0	* 150	29.35	26.78
413	Adenoleukodystrofy	13	* 23.1		
376	Paresthesia	14	* 21.1		
239	Epilepsy, encephalitis	16	* 29.8		
7	SSPE	17	* 48.1		
139	Cerebellitis (Mycoplasma p.)	18	* 19.7		
180	Herpes encephalitis	19	* 14.1		
206	GBS	20	* 15.9		
140	TC ?	21	* 14.2		
228	Ishernic cerebral infarct	22	* 150		

197	Kawasaki	24	* 27.8
133	MS	24	* 37.1
212	Alcohol PNP	25	* 14
66	MS ?	25	* 14
143	Myopathy	25	* 18.3
82	MS	26	* 20.2
258	Guillian-Barré	26	* 14.4
105	MS	26	* 18.4
169	Dementia ?	27	* 37.5
351	Viral meningitis	28	* 17.3
213	MS	28	* 15.7
253	Encephalitis viral ?	28	* 38.1
236	Migraine	29	* 17.3
405	Guillian-Barré	29	* 47.6
72	MS	29	* 14
234	MS	29	* 19.9
128	GBS	29	* 14
138	PNP	32	* 17.6
218	Empty sella	35	* 14
135	MS	35	* 16.3
346	Neuritis optico	35	* 14
117	Trigeminus neurology	36	* 14

350	MS	37	* 14
384	Guillian-Barré	37	* 14
123	CVA	38	* 14
56	TIA (transient ischemic attack)	39	* 14.9
172	CVA ? psych.	40	* 21.7
189	Hemicranial headache	41	* 15.9
119	Amyloidosis	42	* 15.3
69	Meningitis	42	* 26.5
231	Contuno alcohol	42	* 15.5
130	External oftalmoplegia	43	* 53.6
357	CVA	44	* 16.6
391	MS	44	* 14
62	MS ??	44	21.2
124	Syphilis	45	* 20.4
89	PNP	45	* 27.1
122	Cauda equina syndroom etiol.	46	* 14
249	MS	47	* 14
112	MS ?	48	* 14.9
363	Encephalitis	48	* 35.9
125	MS	48	* 24.9
205	GBS	49	
388	MS	50	* 14

418	Tetanos	50	* 14
207	GBS ?	50	* 70.3
114	Cellebella atrofy	51	* 51.5
121	Syphilis	51	* 28.1
35	MS + PNP (diabetic)	51	* 17.3
215	OLM	52	* 14
101	Brain infarct	53	* 52.8
255	PNP + (MS ?), diabetic	53	* 18.4
173	Lyme disease	54	* 17.5
360	(Borrelia) (MS-like)	54	* 34.7
374	ALS	54	* 65.3
179	Lyme disease	54	* 16.4
50	Epilipsy-alcoholism	55	* 21.5
184	Epilepsia	55	* 16.7
210	MS	56	* 16.2
58	CVA	57	* 25.3
137	Pick ?	57	* 77.4
131	Meningial bleeding	57	* 66.7
398	Meningoencephalis	58	* 117.8
349	Meningoencephalis	58	* 35.6
244	Facialis parese	58	* 18.5
219	Pseudobullair syndrome	58	* 39.6

64	MS	58	* 40.7		
36	TIA	59	* 14		
240	Guillian-Barré	68?	* 14	25.06	15.69
70	N. ulnaris/Parkinson (?)	60	* 23.8		
166	tbc	60	* 14		
85	Aneurism	60	* 18.3		
204	Bipyramidal idiop	60	* 16.8		
167	Cerebellar atrophy	61	* 14		
157	Bulbar paralysis	62	* 17.4		
30	Steele richardson	62	* 19.2		
153	Lymfoa CSZ	62	* 22.2		
414	Subacute polyneuropathy	62	* 14		
37	OPCA	63	* 26.7		
109	Pick; ALS	63	* 40.5		
277	COLD	64	* 14		
195	E, dialysis	64	* 14		
148	MS	65	* 15.4		
375	Polyneuropathy, Charot-Marie-Tooth	65	* 15.5		
182	ALS	65	* 14		
230	Parkinson	66	* 21.1		
409	Multiple vascular (pons.)	66			
170	Temp. E	66	* 24.2		

186	ALS	66	* 21.2
59	Menigeal aneurysma	67	* 28.4
233	Korsakow-like post trauma	67	* 29.6
120	ALS	67	* 25.7
550	PNP, CVA, diabetis	67	* 64.2
110	Diabetis	67	* 14
259	Polyvascular syndrome + demyelinis	68	
248	(?)	68	* 14
235	CVA	68	* 77.9
362	Guillain-Barré	68	* 14
208	PNP-zona	68	* 28.8
115	Subacute combined degeneration	68	* 14
98	TIA	68	* 34.9
193	GBS	68	* 44.1
222	Guillain-Barré	69	* 14.1
102	PNP	70	* 14
242	Bipyramidal syndrom PNP atio	72	* 20.8
251	Trauma cerebri commoti	77	* 14
422	Infarct	78	* 20.7
42	Multi-infarct dementia	78	* 38.9

93	Diabetis, PNP, radiculopathy	85	* 61.9		
53	Mixed dementia, Parkinson	85	* 59.3		

Abbreviations: SSPE: Subacute sclerosing panencephalitis, GBS: Guillain-Barré syndrome, TC: Meningeal tuberculosis, MS: Multiple sclerosis, PNP: Polyneuropathy, CVA: cerebrovascular amyloidosis, ALS: Amyotrophic lateral sclerosis, TIA: Transient ischemic attack, OPCA: Olivo-ponto cerebellar atrophy, COLD: Chronic obstructive lung disease.

Example V : Definition of the AT120 epitope

Since AT120 reacts equally well with all isoforms of tau (Goedert et al., 1989), the smallest recombinant tau form was used for deletion mapping. To this, two sites were used for deletion construction, the *SacII* site at position 155 of the human tau 34 sequence and the *SmaI* site at position 220 (Goedert et al., 1989). mTNF-fusion vector pmTNF(MPH) (Innogenetics, Ghent, Belgium), in which the smallest tau open reading frame was fused to 25 amino acids of mouse tumor necrosis factor was cut with *ApaI - SacII*, blunted with T_4 DNA polymerase and ligated. After cutting the ligated material with Sac II and Apa I to reduce non-mutant background, the mixture was transformed into MC1061 pc I587 (Casadaban & Cohen, (1980)). Each selected clone was further characterized by restriction digestion and by its reactivity with anti-tau antibodies.

The same mouse TNF fusion tau vector was used to insert a frame shift mutation in the Xmal restriction site. The vector was cut with *SmaI*, blunted with T_4 DNA polymerase and the ligated mixture was retreated with *SmaI* before transformation in order to reduce non-mutant background. The reactivity pattern of each of the mutants was checked with AT120 monoclonal antibodies by means of Western Blotting. This allowed to localize the epitope of AT120 in a first approximation to the region of 65 amino acids at amino acid 155-221 (Fig.4). However, since in this region also two other antibodies, BT2 (Mercken et al, 1992a) and HT7 (Mercken, Ph.d. Thesis) show reactivity it was mandatory to prove that no competition binding was observed between the latter monoclonal antibodies and AT120.

Therefore, a competition ELISA with each of these antibodies was performed. To this, affinity purified rabbit anti-human tau polyclonal antibodies were coated overnight at 4°C in coatingsbuffer (10 mM Tris pH 8.6, 10mM NaCl, 10mM NaN_3), and after blocking with

0,1 % caseine in PBS 100 µl/well of pure PHF-tau was added for 1 h at 37°C. After washing, 50µl of either AT8, BT2 or AT120 unlabeled monoclonal antibody was added at a concentration of 10µg/ml and incubation was continued for 30 min at 37°C. Next, 50 µl of biotinylated monoclonal antibody was added. Each of these biotinylated antibodies was used in a preset concentration, which in a tau Sandwich ELISA, as described in Example II, gave 50% of the maximal OD value. After a subsequent incubation of 1 h at 37°C, the plates were further treated as described in Example II.

Table III

BIOTINYLATED	Unlabeled competitor Absorbance		
	AT8	AT120	BT2
AT8	0.001	0.372	N.D.
AT120	0.476	0.001	N.D.
BT2	N.D.	0.543	0.054

N.D. refers to not determined absorbances

The results, shown in Table III clearly indicate that the epitope recognized by the monoclonal antibody AT120 is different from the epitopes recognized by the monoclonal antibodies BT2 and AT8. On the basis of their respective reactivity patterns, obtained by incubating solid phase immobilized synthetic nonapeptides, the sequence of which was derived from the epitope of the invention, with each of these monoclonal antibodies followed by visualization of the complexes as in Example I, section.3, it was confirmed that the epitope recognized by AT120 is different from that of each of the other monoclonal antibodies (Table IV and Fig.5)

Table IV. Reactivity of monoclonal antibodies BT2, HT7 and AT120 with solid phase bound nonapeptides.

The nonapeptides are designated in the one-letter amino acid code.

Nonapeptides	Detecting Monoclonal antibody Absorbance		
	HT7	BT2	AT120
GAAPPGQKG (SEQ ID NO 2)	3.00	0.168	0.089
GDRSGYSSP (SEQ ID NO 3)	0.493	3.00	0.553

REFERENCES

Barton A, Harrison P, Najlerahim A, Heffernan J, McDonald B, Robison J, Davies D, Harrison W, Mitra P, Hardy J, Pearson R, (1990) Increased tau messenger RNA in Alzheimer's disease hippocampus. Am J Pathol, 137:497-502.

Baudier J, Cole R (1987) Phosphorylation of Tau proteins to a state like that in Alzheimer's brain is catalyzed by a calcium/calmodulin-dependent kinase and modulated by phospholipids. J Biol Chem 262:17577-17583.

Biernat J, Mandelkow M, Schoter C, Lichtenberg-Kraag B, Steiner B, Berling B, Meyer H, Mercken M, Vandermeeren M, Mandelkow E, The switch of tau protein to an Alzheimer-like state includes the phosphorylation of two serine-proline motifs upstream of the microtubule binding region. EMBO J, 1992, 11:1593-1597.

Binder L, Frankfurter A, Rebhun L (1985) The distrubution of Tau in the mammalian central nervous system. J Cell Biol 101:1371-1378.

Bobrow M, Harris T, Shaughnessy K, Litt G (1989) Catalyzed reporter deposition, a novel method of signal amplification. Application to immunoassays. J Immunol Meth 125:279-285.

Bré M, Karsenti E (1990) Effects of brain microtubule-associated proteins on microtubule dynamics and the nucleating activity of centrosomes. Cell Motil Cytoskeleton 15:88-98.

Brion J, Couck A, Passareiro E, Flament-Durand J (1985) Neurofibrillary tangles of Alzheimer's disease: an immunohistochemical study. J Submicrosc Cytol 17:89-96.

Casadaban M, Cohen S (1980), Analysis of gene control signals by DNA fusion and cloning in Eschericia coli. J Mol Biol, 138:179-207.

De Bont H, Van Boom J, Liskamp R (1990) Automatic synthesis of phosphopeptides by

phosphorylation on the solid phase. Tetrahedron Lett 31:2497-2500.

Delacourte A, Vermersch P (1992) Abnormally phosphorylated tau proteins in Alzheimer's disease: a diagnostic test ? Breakthroughs in Alzheimer's disease, 5-6 March, London, p. 1-4.

Delacourte A, Flament S, Dibe E, Hublau P, Sablonniere B, Hemon B, Sherrer V, Defossez A (1990) Pathological proteins Tau64 and 69 are specifically expressed in the somatodendritic domain of the degenerating cortical neurons during Alzheimer's disease. Acta Neuropathol 80:111-117.

Flament S, Delacourte A, Hemon B, Defossez A (1989) Characterization of two pathological Tau protein variants in Alzheimer brain cortices. J Neurol Sci 92:133-141.

Flament S, Delacourte A (1990) Tau Marker? Nature 346:6279.

Flament S, Delacourte A, Mann D (1990) Phosphorylation of tau proteins: a major event during the process of neurofibrillary degeneration. A comparitive study between Alzheimer's disease and Down's syndrome. Brain Res 516:15-19.

Ghanbari H, Kozuk T, Miller B, Riesing S (1990) A sandwich enzyme immunoassay for detecting and measuring Alzheimer's disease-associated proteins in human brain tissue. J Clin Laboratory Anal 4:189-192.

Goedert M, Wishik C, Crowther R, Walker J, Klug A, Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease : identification as the microtubuli-associated protein tau. Proc Natl Acad Sci USA, 1988, 85, 4051-4055

Goedert M, Spillantini M, Jakes R, Rutherford D, Crowther R (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron 3:519-526.

Goedert M, Spillantini M, Jakes R (1991) Localization of the Alz-50 epitope in recombinant human microtubule-associated protein tau. Neurosci Lett. 126:149-154.

Goedert M, Cohen E, Jakes R, Cohen P (1992) p42 Map kinase phosphorylation sites in microtubule-associated protein tau one dephosphorylated by protein phosphatase 2A1 : implications for Alzheimer's disease. FEBS Lett. 312:95-99.

Greenberg S, Davies P (1990) A preparation of Alzheimer paired helical filaments that displays distinct tau proteins by polyacrylamide gel electrophoresis. Proc Natl Acad Sci USA 87:5827-5831.

Harrington C, Edwards P, Wischik C (1990) Competitive ELISA for the measurement of tau protein in Alzheimer's disease. J Immunol Methods 134:261-271.

Himmler A (1989) Structure of the bovine Tau gene: alternatively spliced transcripts generate a protein family. Mol Cell Biol 9:1389-1396.

Hsu S, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577-580.

Iqbal K, Zaidi T, Thompson C, Merz P, Wisniewski H (1984) Alzheimer paired helical filaments: bulk isolation, solubility, and protein composition. Acta Neuropathol 62:167-177.

Ishiguro K, Ihara Y, Uchida T, Imahori K (1988) A novel tubulin-dependent protein kinase forming a paired helical filament epitope on tau. J Biochem 104:319-321.

Khatoon S, Grundke-Iqbal I, Iqbal K (1992) Brain levels of microtublule-associated protein tau are elevated in Alzheimer's disease : a radioimmuno-slot-blot assay for nanograms of the protein. J Neurochem 59:750-753.

Kosik K, Orecchio L, Binder L, Trojanowski J, Lee V, Lee G (1988) Epitopes that span the

Tau molecule are shared with paired helical filaments. Neuron 1:817-825.

Kosik K, Candall J, Mufson E, Neve R (1989) Tau in situ hybridization in normal and Alzheimer brain: A predominant localization in the neuronal somatodendritic compartment. Ann Neurol 26:352-361.

Ksiezak-Reding H, Dickson D, Davies P, Yen S (1987) Recognition of tau epitopes by anti-neurofilament antibodies that bind to Alzheimer neurofibrillary tangles. Proc Natl Acad Sci USA 84:3410-3414.

Ksiezak-Reding H, Chien C, Lee V, Yen S (1990) Mapping of the Alz50 epitope in microtubule-associated proteins tau. J Neurosci Res 25:412-419.

Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495-497.

Laemmli U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lee V, Balin B, Otvos L, Trojanowski J (1991) A68: a major subunit of paired helical filaments and derivatized forms of normal tau. Science 251:675-678.

Lewis S, Wang D, Cowan N (1988) Microtubule-associated protein MAP2 shares a microtubule binding motif with Tau protein. Science 242:936-939.

Lindwall G, Cole R (1984) The purification of tau proteins and the occurrence of two phosphorylation states of tau in brain. J Biol Chem 259:12241-12245.

Mehta P, Thal L, Wisniewski H, Grundke-Iqbal I, Iqbal K (1985) Paired helical filament antigen in CSF. The Lancet 2:35.

Mercken M, Vandermeeren M, Lubke U, Six J, Boons J, Vanmechelen E, Van de Voorde

A, Gheuens J (1992a) Affinity purification of human tau proteins and the construction of a sensitive sandwich enzyme-linked immunosorbent assay for human tau detection. J Neurochem 58:548-553.

Mercken M, Vandermeeren M, Lubke U, Six J, Boons J, Van de Voorde A, Martin JJ, Gheuens J (1992b) Monoclonal antibodies with selective specificity for Alzheimer Tau are directed against phosphatase-sensitive epitopes. Acta Neuropathol 84:265-272.

Nukina N, Kosik K S, Selkoe D (1987) Recognition of Alzheimer paired helical filaments by monoclonal neurofilament antibodies is due to crossreaction with tau protein. Proc Natl Acad Sci USA 84:3415-3419.

Nukina N, Kosik K, Selkoe D (1988) The monoclonal antibody, Alz 50, recognizes tau proteins in Alzheimer's disease brain. Neurosci Lett 87:240-246.

Papasozomenos S, Binder L (1987) Phosphorylation determines two distinct species of tau in the central nervous system. Cell Motility Cytoskeleton 8:210-226.

Sternberger LA, Hardy PH, Cuculis PH, Meyer HG (1970) The labeled antibody enzyme method of immunohistochemistry : preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315-333.

Steiner B, Mandelkow E, Biernat J, Gustke N, Meyer H, Schmidt B, Mieskes G, Soling H, Drechsel D, Kirschner M, Goedert M, Mandelkow E (1990) Phophorylation of microtubule-associated protein tau: identification of the site for Ca^{2+}-calmodulin dependent kinase and relationship with tau phosphorylation in Alzheimer tangles. The EMBO J 9:3539-3544.

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins form polyacrylamide gels to nitrocellulose sheets : procedure and some applications. Proc Natl

Acad Sci USA 76:4350-4354.

Vallee R (1982) A taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins (MAPs). J Cell Biol 92:435-442.

Weingarten M, Lockwood A, Hwo S, Kirschner M (1975) A protein factor essential for microtubule assembly. Proc Natl Acad Sci USA 72:1858-1862.

Wischik C, Novak M, Edwards P, Klug A, Tichelaar W, Crowther R (1988) Structural characterization of the core of the paired helical filament of Alzheimer disease. Proc Natl Acad Sci USA 85:4884-4888.

Wolozin B, Davies P (1987) Alzheimer-related neuronal protein A68: specificity and distribution. Ann Neurol 22:521-526.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Innogenetics sa.
(B) STREET: Industriepark-Zwijnaarde 7, box 4
(C) CITY: Ghent
(E) COUNTRY: Belgium
(F) POSTAL CODE (ZIP): B-9052
(G) TELEPHONE: 00 32 9 241 07 11
(H) TELEFAX: 00 32 9 241 07 99

(ii) TITLE OF INVENTION: Monoclonal antibodies directed against the microtubule-associated protein tau, hybridomas secreting these antibodies, antigen recognition by these monoclonal antibodies and their applications

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Arg Gly Ala Ala Pro Pro Gly Gln Lys Gly Gln Ala Asn Ala Thr Arg
1               5                  10                  15

Ile Pro Ala Lys Thr Pro Pro Ala Pro Lys Thr Pro Pro Ser Ser Gly
            20                  25                  30

Glu Pro Pro Lys Ser Gly Asp Arg Ser Gly Tyr Ser Ser Pro Gly Ser
        35                  40                  45

Pro Gly Thr Pro Gly Ser Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro
    50                  55                  60

Pro Thr Arg
65
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gly Ala Ala Pro Pro Gly Gln Lys Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Gly Asp Arg Ser Gly Tyr Ser Ser Pro
1 5

CLAIMS

1. Monoclonal antibody which forms an immunological complex with an epitope of an antigen belonging to human normal as well as abnormally phosphorylated tau protein, with said tau protein being liable to be obtained from a brain homogenate, itself isolated from the human cerebral cortex, characterized by the fact that:

- it does not form an immunological complex with other phosphorylated proteins such as MAP-1, MAP-2 and neurofilaments which share part of their sequence with tau protein, as determined by means of an ELISA,

- and it is liable to detect human normal as well as abnormally phosphorylated tau protein in cerebrospinal fluid (CSF), with said tau protein being at a concentration as low as 1.0 pg/ml,

- and it is liable to detect said tau proteins with 100% recovery upon the addition of a fixed amount of tau proteins in tau-protein-negative CSF.

2. Monoclonal antibody according to claim 1, characterized by the fact it forms an immunological complex:

- either with an epitope located within the following amino acid sequence of human tau protein

NH_2- Arg Gly Ala Ala Pro Pro Gly Gln Lys Gly Gln
155 160 165
Ala Asn Ala Thr Arg Ile Pro Ala Lys Thr Pro Pro
170 175
Ala Pro Lys Thr Pro Pro Ser Ser Gly Glu Pro Pro
180 185
Lys Ser Gly Asp Arg Ser Gly Tyr Ser Ser Pro Gly
190 195 200

Ser Pro Gly Thr Pro Gly Ser Arg Ser Arg Thr Pro
205 210
Ser Leu Pro Thr Pro Pro Thr Arg (SEQ ID NO 1)

215 220

- or with any other peptide capable of forming an immunological complex with a monoclonal antibody, which itself is liable to form a complex with an epitope located in the following tau protein region as shown in SEQ ID NO 1.

3. Monoclonal antibody according to claims 1 or 2 secreted by the hybridoma deposited at ECACC on October 8, 1992 under No. 92100853.

4. Hybridoma, which secretes a monoclonal antibody according to anyone of claims 1 to 3, more particularly the hybridoma deposited at ECACC on October 8, 1992 under No. 92100853.

5. Peptides which can be obtained from a brain homogenate, itself isolated from the human cerebral cortex or from cerebral cortex obtained from a patient having Alzheimer's disease and which forms an immunological complex with the monoclonal antibody according to anyone of claims 1 to 3.

6. Peptides liable to form an immunological complex with any of the monoclonal antibodies, according to anyone of claims 1 to 3,

- which are contained in, or are constituted by parts of the sequence as shown in SEQ ID NO 1,

- which contain or are constituted by the sequence of the peptides liable to form an immunological complex with a monoclonal antibody according to anyone of claims 1 to 3, which itself is liable to form a complex with the epitope located in the tau protein region as shown in SEQ ID NO 1.

7. Peptides of about 100 amino acids

- which contain the sequence as shown in SEQ ID NO 1,

- which contain the sequence of the peptides liable to form an immunological complex with a monoclonal antibody according to anyone of claims 1 to 3, which itself is liable to form a complex with a peptide within sequence as shown in SEQ ID NO 1.

8. Peptides according to anyone of claims 5 to 7, which are liable to generate a monoclonal antibody according to anyone of claims 1 to 3 upon immunization.

9. Peptides which are contained in the brain, in the cerebrospinal fluid, or the serum of a patient having Alzheimer's disease or any brain disease involving PHF or normal tau protein and which forms an immunological complex with a monoclonal antibody according to anyone of claims 1 to 3.

10. Process for obtaining and isolating a hybridoma according to claim 3, secreting a monoclonal antibody according to anyone of claims 1 to 3, characterized in that it involves:

- starting from the spleen cells of an animal, e.g. mouse or rat, previously immunized in vivo, or from spleen cells of such cells previously immunized *in vitro* with an antigen recognized by the monoclonal antibody deposited at ECACC on October 8, 1992 under No. 92100853;
- fusing said immunized cells with myeloma cells under hybridoma-forming conditions; and
- selecting those of the hybridomas which secrete the monoclonal antibodies which specifically recognize an epitope of the antigen according to any of claims 5 to 8 and which form an immunological complex with said epitope.

11. Process for producing monoclonal antibodies according to anyone of claims 1 to 3 which involves:

- culturing the selected hybridomas according to claim 4, in an appropriate medium culture; and
- recovering the monoclonal antibodies excreted by said selected hybridomas; or alternatively:
- implanting the selected hybridomas of claim 4 into the peritoneum of a mouse and, when ascites has been produced by the animal, recovering the monoclonal antibodies then formed from said ascites.

12. Process for the detection or diagnosis *in vitro* of brain disease involving either or both PHF-tau or normal tau protein, e.g. Alzheimer's disease, which involves:

- contacting a monoclonal antibody according to anyone of claims 1 to 3, with a preparation of NFT or a detergent-extracted brain homogenate isolated from a patient having had Alzheimer's disease or any other disease involving tau protein or abnormally phosphorylated tau protein under conditions suitable for producing an antigen-antibody complex; and

- separating the antigen from said complex and recovering the antigen sought in a purified form.

13. Process for the detection or diagnosis *in vitro* of brain disease involving abnormally phosphorylated tau protein, e.g. Alzheimer's disease, which includes:

- bringing a sample of brain homogenate, or of cerebrospinal fluid, or of serum from a patient suspected of suffering from a neurological disorder involving tau protein or PHF, more particularly Alzheimer's disease, into contact under *in vitro* conditions with a monoclonal antibody according to anyone of claims 1 to 3, with said conditions being suitable for producing an antigen-antibody complex; and

- detecting the immunological binding of said antibody to said sample of brain homogenate, or of cerebrospinal fluid, or of serum.

14. Process for the detection or diagnosis *in vitro* of brain diseases involving PHF and/or normal tau protein, e.g. Alzheimer's disease, comprising the steps of :

- bringing a sample of unconcentrated cerebrospinal fluid sample isolated from a patient suspected to suffer from a neurological disorder involving normal or abnormally phosphorylated tau protein, more particulary Alzheimer's disease, into contact under *in vitro* conditions with a monoclonal antibody according to anyone of claim 1 to 3, under conditions suitable for producing an antigen-antibody complex;

and,

- detecting the immunological binding of said antibody to said sample of cerebrospinal fluid by means of a sandwich ELISA, preferably by applying the catalysed reporter diagnosis enhancement (CARD) procedure.

15. Kit for the diagnosis *in vitro* of one of the following diseases: Alzheimer's disease, Down's syndrome, Pick's disease, SSPE and other neurological disorders in which

normal tau protein or abnormally phosphorylated tau protein or paired helical filaments are implicated. characterized in that the kit comprises:

- at least a microplate for deposition thereon of any monoclonal antibody according to anyone of claims 1 to 3;

- a preparation containing the sample to be diagnosed *in vitro*, possibly together with a labeled peptide containing the epitope of the invention and preferably with a peptide lying with as shown in SEQ ID NO 1;

- a second antibody

* which can be a monoclonal antibody recognizing an epitope of normal tau, or of abnormally phosphorylated tau protein, or of any peptide according to anyone of claims 5 to 9, with said epitope being different from the one of the invention, or

* which can be a polyclonal antibody recognising normal tau or abnormally phosphorylated tau or a peptide according to anyone of claims 5 to 9, with said polyclonal antibody being liable to form an immunological complex with epitopes which are all different from the epitope of the invention, with said polyclonal antibody being preferably purified by immunoaffinity chromatography using immobilized tau protein, or

- a marker either for specific tagging or coupling with said second antibody;

- appropriate buffer solutions for carrying out the immunological reaction between the monoclonal antibody of the invention and a test sample on the one hand, and the bound second antibody and the marker on the other hand,

- possibly a peptide according to anyone of claims 5 to 9 for standard purposes, or for competition purposes with respect to the antigen which is sought.

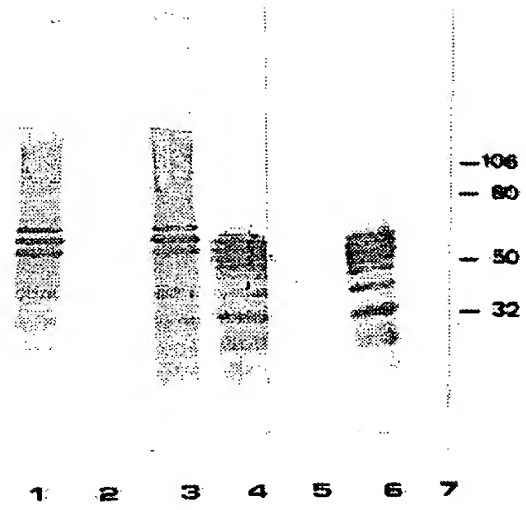

Figure 1

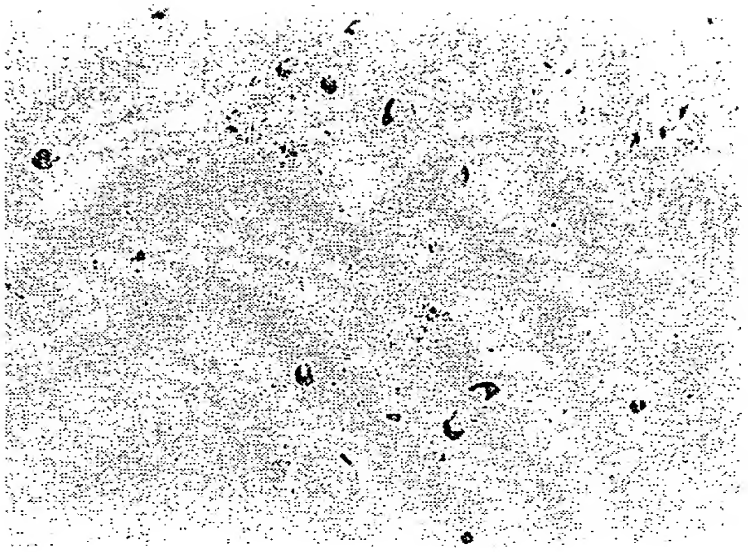

Figure 2A

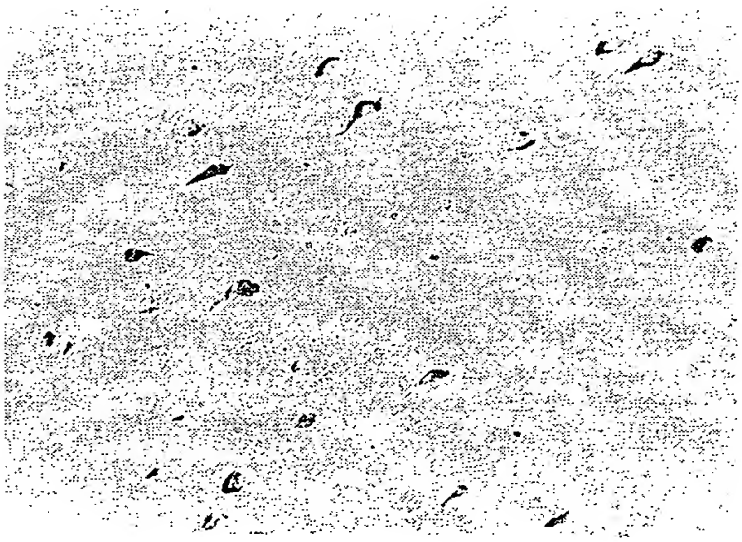

Figure 2B

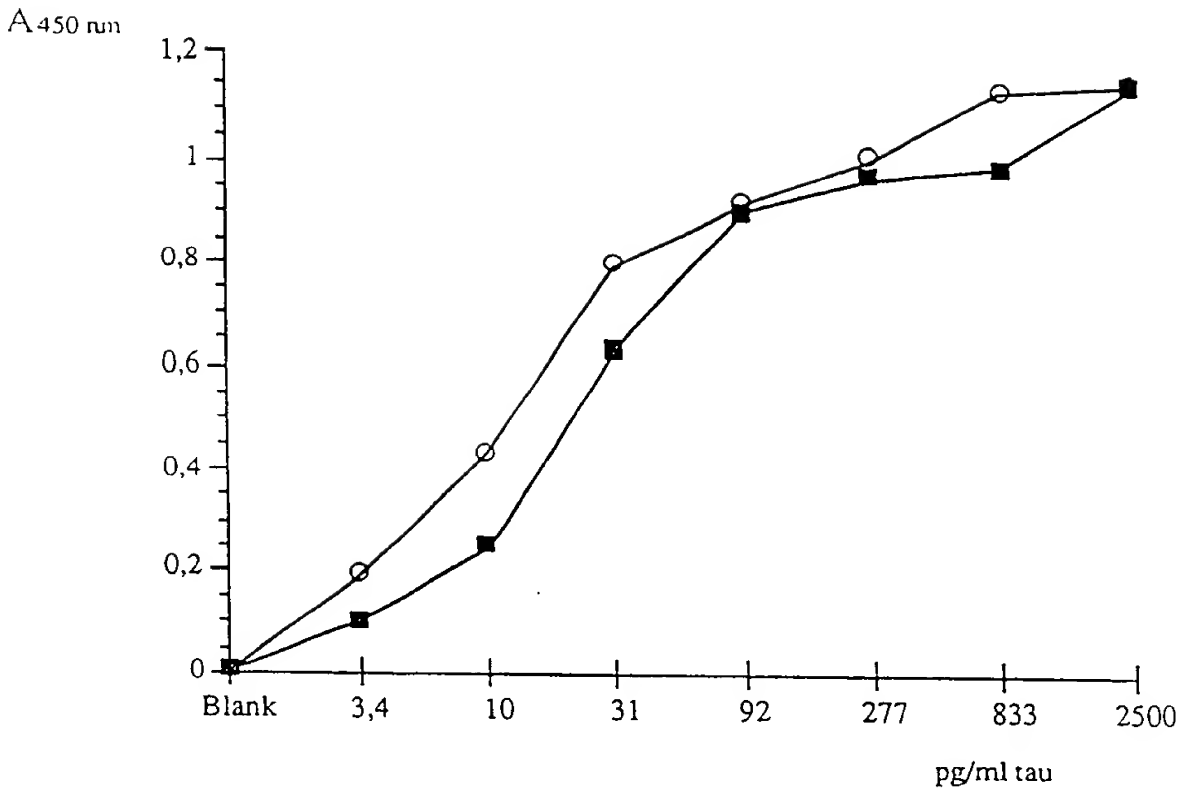

Figure 3

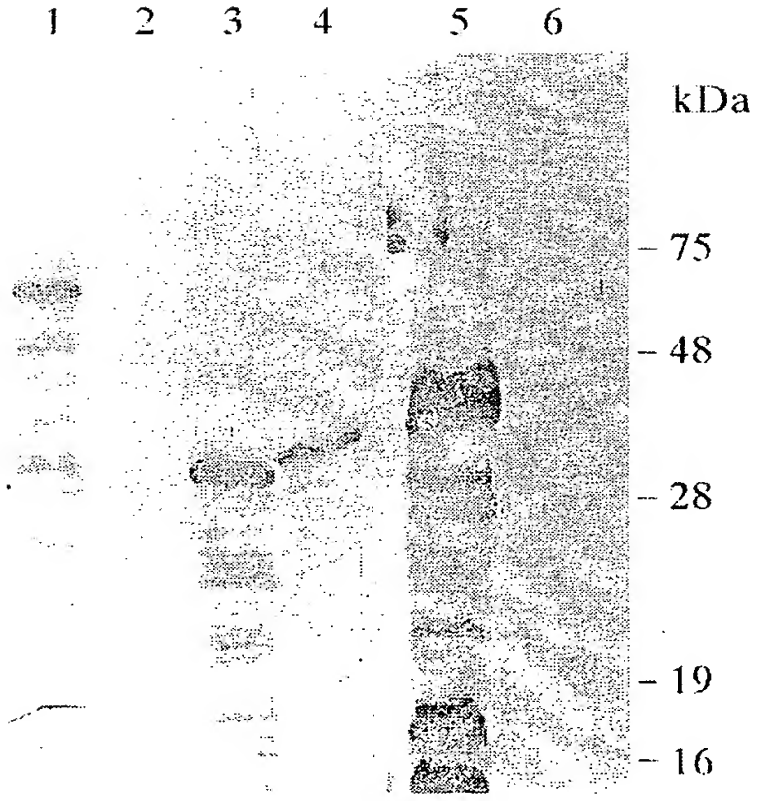

Figure 4

Figure 5

INTERNATIONAL SEARCH REPORT

nal Application No
PCT/EP 93/03499

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/06 C12P21/08 C12N5/20 C07K15/00 G01N33/577
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C12P C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA vol. 88, no. 10 , 15 May 1991 , WASHINGTON DC, US pages 4543 - 4547 S. PAPASOZOMENOS ET AL. 'Altered phosphorylation of tau protein in heat-shocked rats and patients with Alzheimer disease.' see page 4543, left column, line 21 - line 22 see page 4544, right column, line 6 - line 9 --- -/--	1-4, 10-15

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 1994	28. 04. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE EMBO JOURNAL vol. 8, no. 2 , January 1989 , OXFORD, GB pages 393 - 399 M. GOEDERT ET AL. 'Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: Differential expression of tau protein mRNAs in human brain.' see figure 1 -----	5-9

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	32622/93	GB	9200520	WO	9314212		
AU	13690/92	CA	2106091	GB	9105420	WO	9216635
AU	74350/91	AU	80956/87	EP	271988	EP	532060
		GB	8626879	JP	63164892	US	5073676
AU	60423/90	BR	9007523	EP	482053	GB	8916213
		WO	9101375				
AU	65338/90	EP	496777	GB	8923716	US	5254800
		WO	9105865	ZA	9008316		
EP	409625	IL	95132	WO	9101373		

END OF ANNEX

Box I **Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This international search report has not established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically

claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X Y	AU,B,60423/90 (627063) (Imperial Chemical Industries PLC) 7 February 1991 (07.02.91)	1,6,11,12,16,25 10,18-23
X Y	AU,A,65338/90 (Imperial Chemical Industries PLC) 2 May 1991 (02.05.91)	1,6,11,12,16,25 10,18-23
X Y	EP,A,409625 (Calgene Inc.) 23 January 1991 (23.01.91)	1,6,11-13,25 10,18-23
PX PY	Gene, Vol. 123 (No. 2), issued 1993 (Elsevier Science Publishers) Genez A et al, "Isolation of a tomato alcohol dehydrogenase 2- encoding CDNA using phage-promoted antibody screening of a plasmid cDNA library", pages 157-164.	18-21 1,6,7,10-13,16,22,23
A	Biochemical Genetics, Vol. 19 (Nos. 3/4), issued 1981 (Plenum Publishing Corp.) Tanksley S and Jones R, "Effects of O_2 stress on tomato alcohol dehydrogenase activity: description of a second ADH coding gene", pages 397-409.	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 93/00654

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[5] C12N 15/11, C12N 15/53, A01H 1/00, A01H 5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: C12N; WPAT, (Derwent database); (CAS database); Keywords: as below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC: AU Database C12N 15/11; Biotechnology Abstracts (BIOT) (Derwent database)

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
WPAT, CASM, BIOT, Keywords: Claims 1-17, 25, 26 - Fruit#, Grape#, Peach:, Plum#, Strawberr:, Promot:, ripen:, soften:; Claims 18-24 - tomato:, dehydrogenase#, reductase#, ADH#

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
PX PY	AU,A,32622/93 (Zeneca Ltd.) 22 July 1993 (22.07.93)	1, 6, 7, 11-13, 16, 25 10, 18-23
X Y	AU,A,13690/92 (Imperial Chemical Industries PLC) 1 October 1992 (01.10.92)	1, 6, 7, 11-13, 16, 25 10, 18-23
X Y	AU,B,74350/91 (633202) (Imperial Chemical Industries PLC) 11 July 1991 (11.07.91)	1, 6, 11-13, 16, 25 10, 18-23

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 March 1994 (28.03.94)	7 April 1994 (7.4.94)

Name and mailing address of the ISA/AU

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